Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4617–4622, May 1996
Cell Biology

The platelet-derived growth factor α-receptor is encoded by a growth-arrest-specific (*gas*) gene

(growth factor/*lacZ* reporter/retrovirus vector)

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Contributed by Stanley N. Cohen, January 5, 1996

ABSTRACT **Using the *Escherichia coli lacZ* gene to identify chromosomal loci that are transcriptionally active during growth arrest of NIH 3T3 fibroblasts, we found that an mRNA expressed preferentially in serum-deprived cells specifies the previously characterized α-receptor (αR) for platelet-derived growth factor (PDGF), which mediates mitogenic responsiveness to all PDGF isoforms. Both PDGFαR mRNA, which was shown to include a 111-nt segment encoded by a DNA region thought to contain only intron sequences, and PDGFαR protein accumulated in serum-starved cells and decreased as cells resumed cycling. Elevated PDGFαR gene expression during serum starvation was not observed in cells that had been transformed with oncogenes *erb*B2, *src*, or *raf*, which prevent starvation-induced growth arrest. Our results support the view that products of certain genes expressed during growth arrest function to promote, rather than restrict, cell cycling. We suggest that accumulation of the PDGFαR gene product may facilitate the exiting of cells from growth arrest upon mitogenic stimulation by PDGF, leading to the state of "competence" required for cell cycling.**

Growth-arrest-specific (*gas*) genes are preferentially expressed when cell division in culture is stopped by serum deprivation or growth to confluence. In principle, *gas* genes could encode products that cause arrested cell growth, or conversely, could be expressed persistently during the growth-arrested state (G_0) because factors required for cell cycling are lacking. Six separate *gas* genes have thus far been isolated from serum-starved NIH 3T3 cells by subtraction hybridization (1); two other *gas* genes, 354–7 (*gas* 7) and 354–6 (*gas* 8), were detected by expression of a chromosomally-inserted retrovirus-based *lacZ* reporter gene following serum starvation of the same cell line (2). Despite evidence implicating *gas* genes in a variety of functions, including the negative control of cell growth (3), microfilament structure and organization (4), neural membrane function (5, 6), and tyrosine kinase receptor activity (7–9), little is known about the exact biological role of any of these genes.

We report here that an additional *gas* gene identified by a modified version (10) of the retrovirus-based gene-trap vector described earlier (2) encodes a previously known and well-characterized gene product, the platelet-derived growth factor (PDGF) α-receptor (PDGFαR) protein. We suggest that expression of PDGFαR, which has an important role in cellular mitogenic responses and early stage embryogenesis following growth arrest (11, 12), may facilitate the cell cycling that occurs following the addition of PDGF.

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MATERIALS AND METHODS

Isolation of NIH 3T3 Cell Clones That Preferentially Express LacZ During Serum Deprivation. The strategy employed was described previously (2), except that a modified retrovirus-based gene-trap vector pZ-3p, which was derived from pNEOPA-3 (10) by insertion of a promoterless *lacZ* gene from pNASSβ (Clontech) at the *Sal*I site, was used. Procedures for cell culture, transfection, and virus infection are described in detail elsewhere (13). Briefly, retrovirus-infected cells resistant to the antibiotic G418 were selected by growth in 10% calf serum (CS) for 14 days and subsequently were transferred to 0.1% CS for 48 hr to arrest further cell growth. Cells expressing *lacZ* were collected by fluorescence-activated cell sorting (FACS) and cultured in medium containing 10% CS; cells in which *lacZ* expression was turned off or decreased were selected by sorting and expanded again. Finally, another round of sorting produced a cell population that preferentially expressed *lacZ* in 0.1% CS. These cells were cloned in 96-well plates for further analysis.

Cloning and Sequence Analysis of cDNA. cDNA corresponding to *lacZ* fusion transcripts was isolated using a 5′ RACE (rapid amplification of cDNA ends) kit (Life Technologies, Gaithersburg, MD) and total RNA (25 μg) prepared from serum-starved *lacZ*-expressing cells; three nested gene-specific primers (2 pmol of each) complementary to the *lacZ* protein-coding sequence (GenBank accession no. K01793: GSP1, nucleotide positions 1511–1535; GSP4, nucleotide positions 1473–1492; and GSP2, nucleotide positions 1451–1431), an anchor primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG), and a universal amplification primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC) were used. The 5′ RACE product was subcloned in a pBluescript plasmid (Strategene), sequenced, and used as a probe. The 5′ RACE product was used to screen 10^6 phage from a λgt22A oligo-dT-primed cDNA library (provided by Annie C. Y. Chang, Stanford University) that had been constructed using a SuperScript-based cDNA kit (Life Technologies) and polyadenylylated mRNA isolated from serum-starved 3T3 cells. The cDNA fragment from a hybridizing λgt22A clone was removed by digestion with *Sal*I and *Not*I, subcloned into *Sal*I–*Not*I sites of pSPNot72 (Promega), and sequenced using a Sequenase kit (Amersham) and T7 and SP6 primers (Q5021/Q5011, Promega). The reverse transcriptase-mediated polymerase chain reaction (RT-PCR) and

Abbreviations: PDGF, platelet-derived growth factor; αR, α receptor; CS, calf serum; FACS, fluorescence-activated cell sorting; RT-PCR, reverse transcriptase-mediated polymerase chain reaction; FDG, fluorescein di-β-D-galactopyranoside; RACE, rapid amplification of cDNA ends.

Data Deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U47923).

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the primers shown in Fig. 2 were used to detect transcripts in infected and uninfected cells.

Analysis of PDGFαR Gene Expression. RNA was isolated from 2×10^6 cells for each time point and separated on 1% formaldehyde agarose gels (Mops) for Northern blot analysis on GeneScreenPlus nylon filters (DuPont/New England Nuclear). Protein from 10^6 cells was separated by SDS/7% PAGE and blotted onto Immobilon polyvinylidene difluoride protein filters (Millipore). Anti-PDGFαR polyclonal antibody (Santa Cruz Biotechnology) and horseradish peroxidase conjugated secondary antibody (Promega) were used according to vendors' instructions. Western blot signals were detected by chemiluminescence (Amersham). For nuclear run-on analysis, RNA in nuclei prepared from a total of 5×10^7 proliferating (log phase growth) cells or from cells serum-starved for 48 hr was labeled with [α-^{32}P]UTP as described (10). Northern and Western blot analyses and *o*-nitrophenyl β-D-galactopyranoside assays were performed by standard procedures (14). RNase protection assays (Ambion) were done according to vendor's instructions. Fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes), a fluorescent substrate for β-galactosidase, was used for FACS analysis as described by the manufacturer.

DNA Determinations. DNA content of cells was determined by propidium iodide-staining and flow cytometry as described (15) using a FACStar^{Plus} instrument (Becton Dickinson).

RESULTS

Isolation and Characterization of Clones Expressing the *lacZ* Reporter Gene During Growth Arrest. Using pZ-3p and FDG–FACS as indicated in Fig. 1*A* and *Materials and Methods*, we identified four cell clones (clones 17, 40, 51, and 83) that synthesized *lacZ*-mRNA and expressed β-galactosidase preferentially during growth arrest induced by either serum deprivation or culture to a state of confluence (Fig. 1 *B* and *C*). Southern blot analysis of genomic DNA isolated from these clones using a [α-^{32}P]labeled *lacZ*- or *aph*–DNA fragment (Fig. 1*A*) as probe revealed a single copy of pZ-3p-derived provirus integrated in each case at the same chromosomal DNA site (13), suggesting that the four isolates are sibs. As the selection procedure used requires insertion of the promoterless reporter gene into a chromosomal gene expressed preferentially during growth arrest, the gene containing the reporter in these clones is by definition a *gas* gene.

The *lacZ* Transcript Was Fused to a 5′-Untranslated Segment of PDGFα Receptor mRNA. 5′ RACE cloning revealed that a novel 111 nt transcript sequence was fused to the *lacZ* reporter gene (Fig. 2). RT-PCR using primers 5′ RAC and GSP5, which are specific to sequences within the 111-nt 5′ RACE cDNA product and *lacZ*, respectively (Fig. 2), showed that a fusion transcript containing both *lacZ* and the 111-nt segment found 5′ to it in the RACE product is made in the *lacZ*-expressing cells (Fig. 3*A*, lanes 5 and 7), but is absent from NIH 3T3 control cells (Fig. 3*A*, lanes 2 and 3). RT-PCR analysis using two primers (5′ RAC and 3′ RAC), located within the 111-nt 5′ RACE sequence showed that transcripts containing the 111-nt sequence exist also in uninfected growth arrested NIH 3T3 cells (Fig. 3*A*, lanes 1, 4, and 6).

Using the 111-nt cDNA segment as a probe, we identified four hybridizing cDNA clones in a cDNA library made from poly(A)$^+$ mRNA isolated from serum-starved 3T3 cells. Southern blotting and partial DNA sequencing showed that all four isolates included a 1.6-kb insert (designated *gas* 9; Fig. 2*B*), which contained a segment identical to nucleotides 176-1780 of the previously reported sequence for murine PDGFαR cDNA (GenBank accession number M84607); termination of the *gas* 9 cDNA fragment at the *Not*I site at position 1780 (Fig. 2*B*) likely resulted from *Not*I-linker digestion during library construction, as there is an internal *Not*I site at that location in the PDGFαR cDNA sequence. The 80 nt at the 5′ end of *gas* 9 (Fig. 2 *Abc* and *B*), which correspond to positions 31–111 of the 5′ RACE product (Fig. 2 *Aab* and *B*),

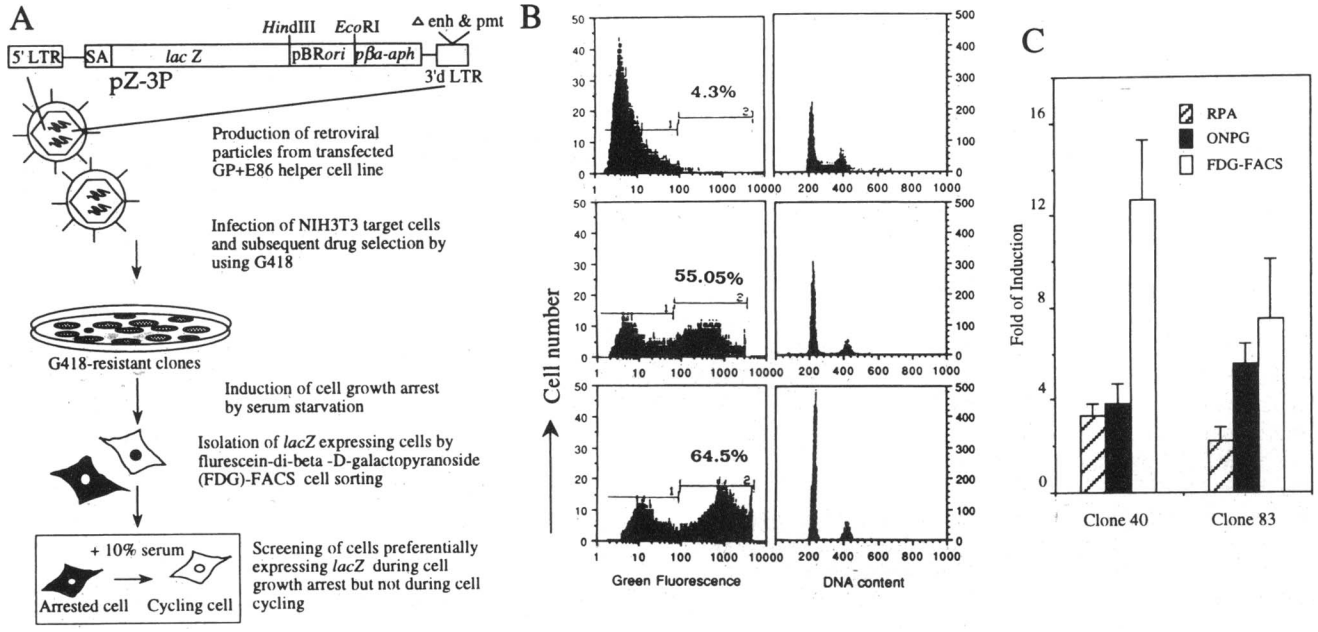

FIG. 1. (*A*) Experimental design. The procedure detects clones that preferentially express *lacZ* during cell growth arrest. *lacZ* expression in either the cytoplasm or nucleus was indicated by dark shading. (*B*) *lacZ* expression induced by serum deprivation in clone 83 is shown. Fluorescence intensity is shown during cultivation in 10% CS at nonconfluent (*Top*), or confluent (*Middle*) cell density, or in 0.1% CS at nonconfluent density (*Bottom*). *lacZ* expression (*Left*) was analyzed by FDG–FACS as described (2); cell cycle distribution (*Right*) was determined using propidium iodine staining and flow cytometry. The horizontal axis shows green fluorescence or DNA content; the ordinate shows the cell number analyzed. Cells showing an intensity of fluorescence below (designated as 1) or above (designated as 2; percentage shown) channel 100 are indicated. (*C*) Serum-deprivation-induced *lacZ* expression is shown for clones 40 and 83. Fold induction equals the amount of *lacZ* expression after 48 hr in 0.1% CS divided by the amount of *lacZ* expression during log-phase growth at nonconfluent density. Bars indicate results of RNase protection assays (RPA), *O*-nitrophenyl-β-D-galactopyranoside (ONPG), and FDG-FACS assays and are the average of three or more analyses.

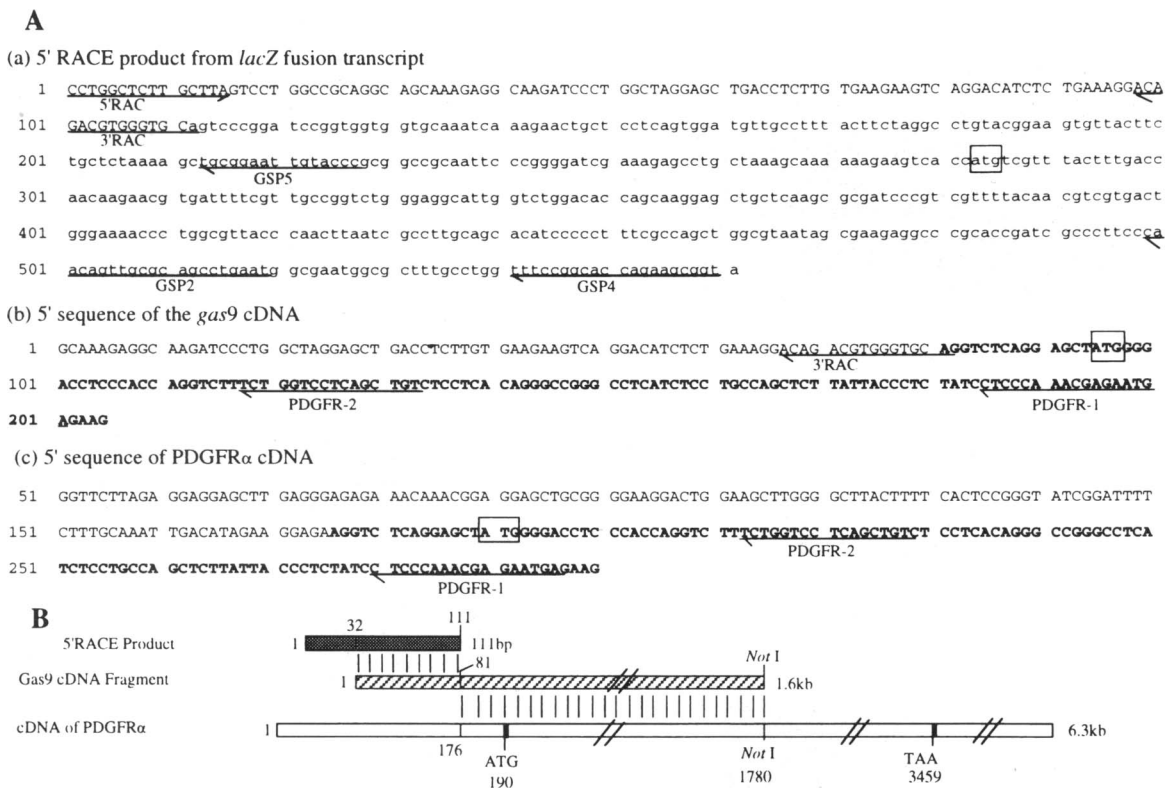

FIG. 2. Sequence and relationship of relevant cDNAs. (*Aa*) Sequence of 111-nt 5′ RACE transcript fused to *lacZ*. Primer locations are indicated. The translation initiation codon for β-galactosidase is boxed. Lowercase and uppercase nucleotides show the cDNA sequence for *lacZ* and the segment fused to its 5′ end, respectively. The *lacZ* gene was derived from pNASSβ (Clontech) and contains a splice acceptor site of simian virus 40 and the *Drosophila melanogaster* alcohol dehydrogenase gene (DmADH) fragment, which provides an in-frame translation initiation codon (boxed). PCR and 5′ RAC and 3′ RAC primers were used to isolate the 111-nt DNA segment fused to *lacZ*; this was the template for synthesis of a random ^{32}P-labeled DNA probe used to isolate *gas* 9 cDNA. (*Ab*) The 5′ sequence of *gas* 9 cDNA, which was isolated from a λgt22A oligo-dT-primed cDNA library as described, is shown; boldface type indicates sequences identical to PDGFαR (GenBank accession no. M84607). The primers 3′ RAC, PDGFR-1, and PDGFR-2 were used for RT-PCR (see Fig. 3). (*Ac*) Sequence at 5′ end of PDGFαR cDNA. The translation initiation codon is boxed. Arrows indicate the direction of extension of individual primers. (*B*) Segments of homology among the 5′ RACE product (black rectangle), *gas* 9 cDNA (hatched rectangle), and PDGFαR cDNA (open rectangle). 5′ Termini are assigned position 1. Identity between cDNAs is indicated by vertical lines; diagonal lines indicate discontinuity. Positions of PDGFαR translation start and stop codons are indicated.

are not present in the previously reported PDGFαR sequence, consistent with the absence of any GenBank sequence that corresponds to the 111-nt 5′ RACE product we found fused to *lacZ*.

To further investigate the relationship of the 111-nt sequence to the known PDGFαR gene sequence, we analyzed RNA isolated from serum-starved naive NIH 3T3 cells using RT-PCR and three separate pairs of primers (Fig. 2*A*) chosen to detect transcripts that contain both the previously determined PDGFαR sequence and the 111-nt sequence identified in the 5′ RACE product. As shown in Fig. 3*B*, three PCR products having the sizes expected from the positions of the primer sets were observed, indicating that the 111-nt sequence is joined to the 5′ end of PDGFαR transcripts made in these cells. Southern blot analysis (Fig. 3*C*) using genomic DNA fragments from the region 5′ to the protein-coding sequence of the PDGFαR gene indicated that the 111-nt transcript segment is encoded by a DNA region located in what previously has been identified as the first intron of the PDGFαR gene. As shown in Figs. 2*B* and 3, position 81 of *gas* 9 cDNA (which is the 3′ end point of the 111-nt 5′ RACE product we identified) corresponds to coordinate 176, which is precisely the splice acceptor site of exon 2 of the previously reported PDGFαR (16) sequence, suggesting that PDGFαR mRNA exists in alternatively spliced forms.

Expression of PDGFαR Is Induced by Cell Growth Arrest and Suppressed by Cell Cycling. To confirm that the PDGFαR gene is expressed preferentially during growth arrest of cells lacking the reporter, we determined the effects of serum deprivation and cell cycle progression on PDGFαR-specific gene expression using Western and Northern blot analyses. As seen in Fig. 4 *A* and *B*, serum starvation resulted in intracellular accumulation of PDGFαR mRNA and protein in NIH 3T3 cells. Addition of 20% CS or of basic fibroblast growth factor, which promotes cycling of serum-starved cells (13, 17) without causing internalization of PDGF receptors (17), resulted in decreased PDGFαR mRNA and protein, respectively (Fig. 4).

Nuclear run-on assays using nuclei prepared from either proliferating or growth-arrested cells showed that the effect of serum starvation on PDGFαR gene expression is controlled at the transcriptional level: the concentration of PDGFαR mRNA increased 4-fold during 24 hr of culture in the absence of serum (Fig. 4*D*); in contrast, in control experiments the concentration of c-*myc* mRNA, which is shut off during growth arrest (18), decreased as expected. Additionally, as shown in Fig. 5, transformed NIH 3T3 cells expressing ErbB2, Raf, or Src, which prevent cell growth arrest during serum deprivation, failed to show consequent induction of PDGFαR expression—indicating that the preferential expression observed for PDGFαR in non-transformed serum-starved cells does not result from serum deprivation *per se*.

DISCUSSION

In the absence of growth factors commonly present in serum, mammalian cells in culture are induced to leave the cell cycle

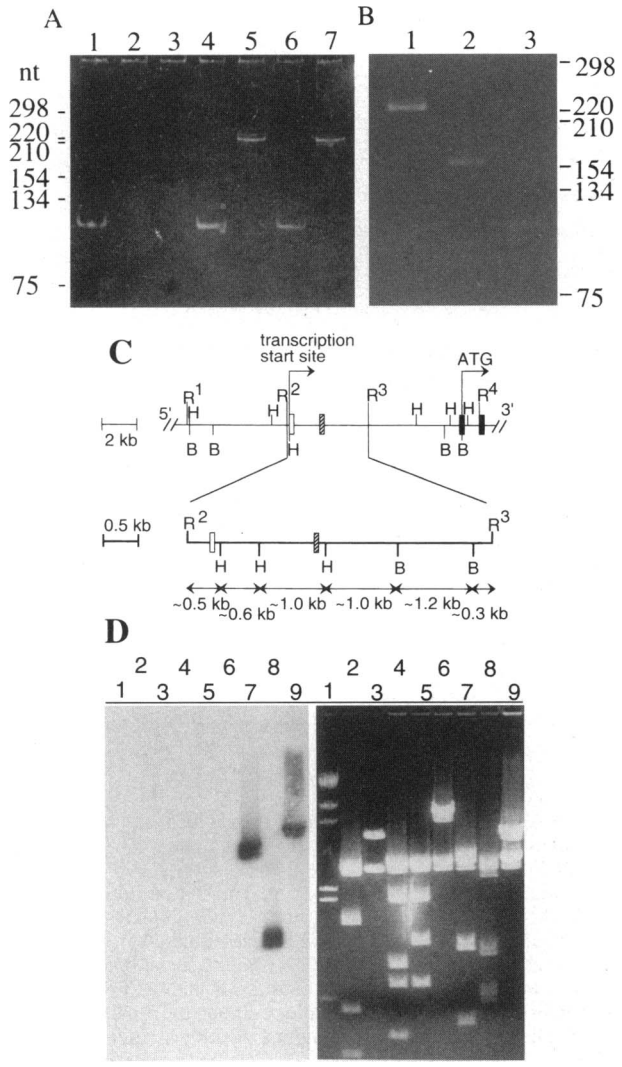

FIG. 3. The 111-nt segment fused to *lacZ* is part of the PDGFαR gene. (*A*) RNA isolated from either naive NIH 3T3 cells (lanes 1–3) or from *lacZ* expressing cell clone 40 (lanes 4 to 5) or 83 (lanes 6 to 7) was amplified by RT-PCR using primer 3′ RAC (lane 1) or GSP5 (lanes 2–7) for first strand cDNA synthesis. Primers for PCR amplification were 3′ RAC and 5′ RAC (lanes 1, 2, 4, and 6) or GSP5 and 5′ RAC (lanes 3, 5, and 7). (*B*) Total RNA extracted from growth-arrested NIH 3T3 cells was primed using random hexamers and reverse transcribed to generate first-strand cDNA. PCR amplification used 5′ RAC and PDGFR-1 (lane 1), 5′ RAC and PDGFR-2 (lane 2), or 5′ RAC and 3′ RAC (lane 3). PCR products were electrophoresed on 1× TBE/6% acrylamide gels and stained with ethidium bromide. Size markers (1-kb ladder, Life Technologies) are shown. (*C*) Diagram of PDGFαR DNA regions upstream of the third exon. Vertical bars represent exon sequences: the open bar indicates the 5′-UTR identified earlier (16), the shaded bar indicates the 111-nt sequence identified in this report, and filled bars represent coding exons (bars are not drawn to scale). Transcription and translation start sites are indicated. R, H, and B are abbreviations for restriction endonuclease sites, *Eco*RI, *Hind*III, and *Bam*HI, respectively. (*D*) Southern blot analysis of pKS$^+$-derived plasmid constructs pKS$^+$58, pKS$^+$5.0, and pKS$^+$8.7 DNA constructs containing PDGFαR R^1-R^2, R^2-R^3, and R^3-R^4 restriction fragments at the *Eco*RI site. Lanes: 1, bacteriophage λ-*Hind*III digest (size markers); 2, pKS$^+$58 by R/H/B; 3, pKS$^+$58 by R/H; 4, pKS$^+$8.7 by R/H/B; 5, pKS$^+$8.7 by R/H; 6, pKS$^+$8.7 by R; 7, pKS$^+$5.0 by R/B; 8, pKS$^+$5.0 by R/H; 9, pKS$^+$5.0 by R. (*Left*) Autoradiogram of DNA electrophoretically separated on a 0.8% TAE agarose gel and probed with 111-nt fragment amplified by PCR and ^{32}P-labeled by random priming (14). (*Right*) The preblotted gel was stained with ethidium bromide.

and enter a state of growth arrest (G_0), where they remain unless stimulated to reenter the cycle by mitogenic signals (18).

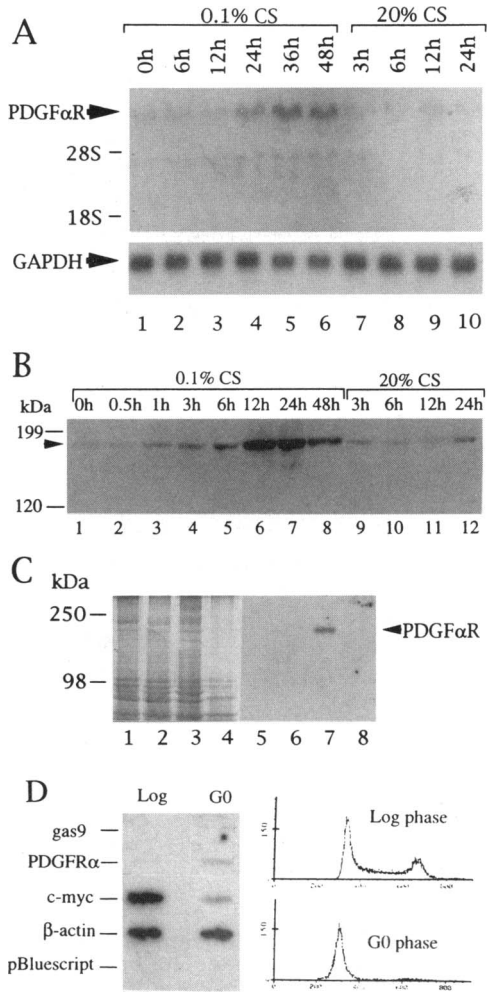

FIG. 4. Analyses of PDGFαR mRNA and protein expression during serum starvation and after reentry into the cell cycle. Northern blot (*A*, 20 μg total RNA per well), Western blot (*B* and *C*, 100 μg total cellular protein per well), and nuclear run-on (*D*) analyses used standard techniques (14). CS concentrations, duration of treatment, and sampling times are shown. Probes were [α-^{32}P]random labeled 1.6-kb *Eco*RI fragment of PDGFαR (coordinates 312-1948) or 1.3-kb full-length cDNA of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH control). Positions of 28S and 18S ribosomal RNA and protein size markers are indicated. (*C*) Lanes 1–4 show Coomassie blue stained gel of total protein; lanes 5–8 show Western blot. Cells cultured for 48 hr in 0.1% CS were then treated for 24 hr with 20% CS (lanes 1 and 5) or basic fibroblast growth factor (30 μg/ml) (lanes 2 and 6). Lanes 3 and 7 show protein from serum-starved cells (0.1% CS) for 48 hr. Lanes 4 and 8 show protein from cells growing exponentially in 10% CS. (*D*) Nuclear run-on analysis. (*Left*) Nuclei were isolated as described from logarithmically growing (Log phase) or serum-starved (G_0 phase) cells for synthesis of ^{32}P-labeled nascent transcripts. In this experiment, each linearized plasmid DNA (20 μg) was denatured in 0.2 M NaOH for 30 min, neutralized in 6× standard saline citrate for 10 min, and transferred to filters using a Schleicher & Schuell slot-blot apparatus. Species of DNA immobilized on the nylon membrane are indicated. (*Right*) DNA content analysis as described in Fig. 1*B*.

Certain genes are known to be preferentially or selectively expressed during growth arrest (1–3, 19–22). In the experiments reported here, we used a Moloney murine leukemia virus-based gene trap vector carrying *lacZ* (cf refs. 2 and 23–25) to search for such genes. Our search identified a novel chromosomally encoded 111-nt transcript sequence that was fused in growth-arrested cells to a splice acceptor site we introduced 5′ to *lacZ*. In a cDNA library constructed from mRNA isolated from naive serum-starved NIH 3T3 fibro-

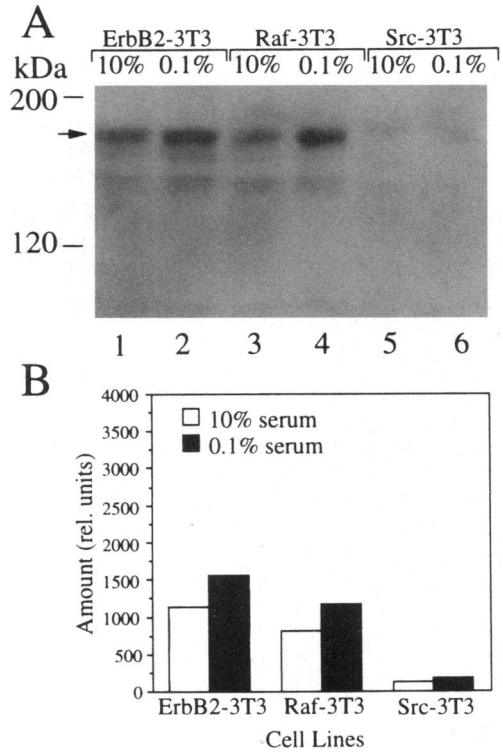

FIG. 5. Expression of PDGFαR protein in oncogene-transformed cells. (*A*) Western blot analysis as described in Fig. 4. Total cellular protein was extracted from v-ErbB2/3T3 (lanes 1 and 2), v-Raf/3T3 (lanes 3 and 4), and v-Src/3T3 (lanes 5 and 6) cells cultured in 10% (lanes 1, 3, and 5) or 0.1% (lanes 2, 4, and 6) CS for 24 hr. (*B*) Expression of PDGFαR protein in oncogene-transformed NIH 3T3 cell relative to nontransformed cells. Band intensity was determined densitometrically.

blasts, the same 111-nt sequence was found spliced to the 5′ end of an exon (exon 2) encoding part of the previously identified PDGFα receptor sequence. Further analysis showed that synthesis of mRNA encoding PDGFαR in parental NIH 3T3 cells is induced by growth arrest, confirming that PDGFαR is a *gas* gene. The 111-nt sequence found fused to the 5′ end of PDGFαR exon 2 is specified by a gene segment thought previously to contain only an intron. The junction of the 111-nt region and the sequence designated earlier as PDGFαR exon 2 occurs precisely at a previously identified splice site, implying that PDGFαR transcripts undergo alternative splicing. Additional RT-PCR analysis (data not shown) indicates that both growth-arrested and cycling cells produce transcripts containing the 111-nt segment fused to exon 2.

The addition of PDGF protein to growth-arrested cells enables entry from G_0 into the G_1 phase of the cell cycle; however, while such PDGF-stimulated cells become "competent" for cycling, progression through G_1 and entry into S phase requires additional factors usually present in serum or in platelet-poor plasma (11, 26). Thus, the mitogenic response results from the combined effects of PDGF-induced competence and "progression factors" such as epidermal growth factor and insulin-like growth factor I (27, 28). PDGFαR protein, which binds to all three isoforms of PDGF (AA, AB, and BB), has a central role in mediating the state of competence induced by PDGF (29). Selective expression of the PDGFαR gene product during G_0 may lead to accumulation of this protein on the surface of growth-arrested cells, consequently facilitating exiting from the G_0 state. The PDGFαR mRNA concentration decreased following stimulation of DNA replication (data not shown) by the addition of 20% CS to serum-deprived cells and remained at a low steady-state level in actively cycling cells (Fig. 4*A*). A similar role in the entry of quiescent cells into G_1 has been proposed for the PDGFβ receptor, which during preparation of this report was reported to also be regulated in a growth-dependent manner in fibroblasts (17).

Our finding that PDGFαR is not induced in serum-deprived NIH 3T3 cells transformed with the oncogenes *erb*B2, *src*, or *raf*, all of which prevent growth arrest under conditions of serum deprivation, indicates that control of PDGFαR expression is governed by cell cycling rather than by the presence or absence of serum growth factors *per se*. Additionally, the discovery that both PDGFαR (Fig. 5) and β receptor (17) proteins are decreased in *src* oncogene-transformed NIH 3T3 cells may explain the reduced number of PDGF-BB binding sites found previously in such cells (30), as well as the lessened ability of PDGF to induce immediate early response genes in oncogene-transformed cells (31, 32). The low abundance of both PDGFα (this work) and β (17) receptors observed in *src*-transformed cells suggests that overexpression of the *src* oncogene may down-regulate PDGFα β receptor gene expression.

Whereas some genes expressed preferentially during the G_0 state assist in maintaining growth arrest and delay the transition from G_0/G_1 to S phase of the cell cycle (3, 32–36), the PDGFαR gene, which we have now shown is a *gas* gene, acts in conjunction with other growth factors to promote cell cycling. The detection, during a search for loci expressed preferentially during growth arrest, of a gene encoding the receptor for a well-characterized and widely studied growth factor, together with the finding that the PDGFβ receptor gene is also preferentially expressed during growth arrest, now raises the possibility that other *gas* genes whose functions are currently unknown also may be positive—rather than negative—regulators of the cell cycle.

We thank A. C. Y. Chang for providing a growth-arrest 3T3 cell cDNA library, L. Eisenbach for a PDGFαR cDNA clone, Yea-Miin Lin for assistance in FACS analyses, and C. K. Chou, Y. H. Sun, B. C. Chung, H. F. Yang-Yen, Y.-H. Wu-Lee, C. C.-K. Chao, and T. C. Chang for critical comments and discussion. This study fulfilled in part requirements for the Ph.D. dissertation of C.-J.L. This work was supported in part by an intramural fund from the Academia Sinica and by grants from the Republic of China National Science Council (Grants NSC83/84-0412/2331-B001-067/044-M15; NSC85-22311-B001-035) to S.L.-C. and Grant NSC 84-2311-B-001-094-Y to S.N.C. C.W. was supported by the American Institute for Cancer Research (Grant 94A78).

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